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L3	L2 and 424/450.ccls.	211	L3
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DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD; PLUR=YES; OP=OR			
L3	(liposome adj10 dialy$) same (ammonium adj1 sulfate)	17	L3
L2	L1 and 424/450.ccls.	289	L2
L1	liposome adj10 (dialy$)	573	L1

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L3: Entry 14 of 17 File: USPT Dec 28, 1999

DOCUMENT-IDENTIFIER: US 6008203 A
TITLE: Methods for treatment of EGF receptor associated cancers

Detailed Description Text (136):
A liposome is prepared by mixing carbohydrate, dipalmitoyl phosphatidylcholine, cholesterol, and an antioxidant such as Vitamin E (7.5:38:23:1 by weight) in methanol:chloroform (1:1) and drying the mixture. 150 MM ammonium sulfate, pH 5.5, is added to the mixture and it is sonicated for 30 minutes to produce liposomes with a range of sizes. Liposomes with a narrow size distribution are isolated by passing this mixture through a polycarbonate filter with 80 to 100 nm pore size twenty to thirty times, to yield liposomes with a similar diameter. The liposomes are then dialyzed with 5% glucose, pH 7, to set up a pH gradient. The daunomycin is incorporated into the liposomes by heating the liposomes at 60.degree. C. for one hr with 3.8 M daunomycin in 5% glucose, pH 7. The preparation is cooled to room temperature and unincorporated daunomycin is removed by ion exchange.

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L3: Entry 13 of 17 File: USPT Apr 18, 2000

DOCUMENT-IDENTIFIER: US 6051251 A
TITLE: Liposome loading method using a boronic acid compound

Detailed Description Text (53):
The liposome suspension was prepared by dissolving the lipids in ethanol and drying the lipids to a thin film. The lipid film was hydrated with an ammonium sulfate solution to form liposomes and then the liposomes were extruded to obtain liposomes of about 100 nm. The liposome suspension was dialyzed against a sucrose solution, thus obtaining liposomes encapsulating a 250 mM ammonium sulfate solution in an external buffer of 10% sucrose at pH 6.5. The total lipid concentration was 52 .mu.moles/ml.

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L4: Entry 90 of 123 File: USPT Aug 17, 1999

DOCUMENT-IDENTIFIER: US 5939096 A
TITLE: Liposome drug-loading method and composition

Abstract Text (1):
A method of stably encapsulating a weak acid drug in liposomes, at a high concentration, is disclosed. The method employs a proton shuttle mechanism involving the salt of a weak acid to generate a higher inside/lower outside pH gradient. The weak acid compound accumulates in liposomes in response to this gradient, and may be retained in the liposomes by cation-promoted precipitation or low permeability across the liposome transmembrane barrier. Also disclosed is a reagent combination for practicing the method, and a liposome composition formed by the method.

Brief Summary Text (38):
In the case of ionizable hydrophilic or amphipathic drugs, even greater drug-loading efficiency can be achieved by loading the drug into liposomes against a transmembrane pH gradient (Nichols, et al., 1976; Cramer, et al., 1977). Typically the drug contains an ionizable amine group, and is loaded by adding it to a suspension of liposomes prepared to have a lower inside/higher outside pH gradient. Although high drug loading can be achieved by this approach (e.g., U.S. Pat. No. 5,077,056), the drug tends to leak out over time as the liposome transmembrane proton gradient decays.

Brief Summary Text (39):
The latter problem has been addressed, for drugs having an ionizable amine group, by loading the drug across an ammonium ion gradient (Haran, et al., 1993). Ammonium ions within the liposomes are in equilibrium with ammonia, which is freely permeable through the liposome membrane, and protons, which therefore accumulate as ammonia is lost from the liposomes, leading to a lower inside/higher outside pH gradient. After establishing the gradient, excess ammonium ions within the liposomes provide a reservoir of protons, to maintain the liposome pH gradient over time. This approach, however, is limited to drugs which are positively charged in their ionized state.

Brief Summary Text (42):
The present invention includes, in one aspect, a method of forming liposomes having a higher inside/lower outside pH gradient. The gradient is established by preparing a suspension of liposomes in an aqueous solution containing a salt of a weak acid which is capable of freely permeating the liposome membrane. The suspension is then treated to produce a higher inside/lower outside concentration gradient of the weak acid. The weak acid is allowed to distribute between inner and outer compartments, acting as an inside-to-outside proton shuttle to generate a higher inside/lower outside pH gradient.

Brief Summary Text (44):
In a related aspect, the invention provides a method for loading a weak-acid compound into liposomes having a higher inside/lower outside pH gradient. Loading is carried out by adding the weak acid compound to a suspension of liposomes having a higher inside/lower outside gradient of a salt of a weak acid which includes the given cation. The protonated form of the weak acid salt acts as an inside-to-

outside proton shuttle to generate a higher inside/lower outside pH gradient to drive loading of the weak acid compound into the liposome interior.

Brief Summary Text (47):
In another general embodiment, the protonated form of the weak acid compound is readily able to permeate the liposome transmembrane barrier only at a temperature above the phase transition temperature of the liposomes. The compound is loaded at a temperature above this phase transition temperature, and the suspension is stored at a temperature below the phase-transition temperature.

Drawing Description Text (5):
FIG. 3 is a schematic illustration of the loading of a weak acid drug, "D--COOH", into liposomes against a higher inside/lower outside pH gradient established by the method of the present invention;

Detailed Description Text (12):
A "higher inside/lower outside pH gradient" refers to a transmembrane pH gradient between the interior of liposomes (higher pH) and the external medium (lower pH) in which the liposomes are suspended. Typically, the interior liposome pH is at least 1 pH unit greater than the external medium pH, and preferably 2-4 units greater.

Detailed Description Text (13):
II. Preparation of pH Gradient Liposomes

Detailed Description Text (14):
This section describes the preparation of a suspension of liposomes having a higher inside/lower outside pH gradient, in accordance with the invention.

Detailed Description Text (19):
Lipids for use in the present invention may be relatively "fluid" lipids, meaning that the lipid phase has a relatively low gel-to-liquid-crystalline phase transition temperature, e.g., at or below room temperature, or alternately, relatively "rigid" lipids, indicating that the lipid has a relatively high gel-to-liquid-crystalline phase transition temperature, e.g., at temperatures up to about 50.degree. C. As a general rule, the more rigid, i.e., saturated lipids, contribute to greater membrane rigidity in the lipid bilayer structure, and thus to more stable drug retention after active drug loading. Preferred lipids of this type are those having phase transition temperatures above about 37.degree. C.

Detailed Description Text (33):
C. Formation of Liposome pH Gradient

Detailed Description Text (37):
After adjusting the external medium to produce a higher inside/lower outside concentration gradient of the weak acid salt, the weak acid is allowed to distribute between inner and outer liposome compartments, with the weak acid salt acting as an inside-to-outside proton shuttle, until an equilibrium higher inside/lower outside pH gradient is formed.

Detailed Description Text (38):
FIG. 1 illustrates the proton-shuttle mechanism by which the pH gradient is formed. The figure shows a liposome 10 having a bilayer membrane 12 and having encapsulated therein, the salt of a weak acid, in this case, the calcium salt of acetate, with the acetate anion being in equilibrium with the uncharged (protonated) form of the acid. The bilayer membrane serves as a partition between the liposome inner compartment, indicated at 14, and an outer bulk phase suspension medium 16.

Detailed Description Text (49):
It will be appreciated from the above that the pH gradient across the liposomes is self-regulating and self-sustaining, i.e., not degraded by leakage of protons into

the liposomes or hydroxyl ions out of the liposomes after the gradient is formed. This feature can be appreciated from the proton shuttle mechanism illustrated in FIG. 1. Here it is assumed that a pH gradient has been established and the liposomes are stored over an extended period in suspension. During storage, as hydroxyl ions leak out from the liposomes into the external medium, and as protons leak into the liposomes from the external medium, the equilibrium between charged and protonated form of the weak acid (acetate) in the liposomes shifts toward the protonated form, increasing the level of proton shuttling out of the liposomes, acting to restore the pH gradient.

Detailed Description Text (51):
The pH gradient liposomes formed as above are used in loading a weak-acid compound into the liposomes, according to another aspect of the invention. In this method, the compound is added to a suspension of the pH gradient liposomes, and the suspension is treated under conditions effective to load weak acid compound within the liposomes.

Detailed Description Text (53):
FIG. 3 illustrates the mechanism of drug loading into liposomes, in accordance with the method. The figure shows a liposome 18 having a bilayer membrane 20 and a higher inside/lower outside pH gradient, by virtue of a higher inside/lower outside gradient of a weak acid, i.e., the anion of the weak acid, in this case the acetate anion.

Detailed Description Text (54):
The bottom of the figure shows the mechanism by which a higher inside/lower outside pH gradient is formed, as described in Section II. The upper part of the figure shows the mechanism of loading of the weak acid compound, indicated as "D--COOH". The compound, which is present originally only in the external compartment, is shown in equilibrium in this compartment between negatively charged and uncharged, protonated forms, with lower pH favoring the latter form. As indicated, the compound is able to pass through the liposome membrane only in its protonated form. In the absence of a pH gradient, the compound would equilibrate to equal concentrations on both sides of the membrane. Because of the higher internal pH, the equilibrium between the charged and uncharged form of the compound is shifted toward the charged, nonpermeable form, leading to net loading of the compound in the liposomes. Assuming the compound remains in solution in its liposome-loaded form, the extent of liposome loading for weak acid compounds is governed by the Henderson-Hasselbach relationship:

Detailed Description Text (57):
By way of example, with a pH gradient of 4 pH units, and an outside-to-inside volume ratio of 100:1, a theoretical loading factor of 100:1 inside:outside is possible. Based on these considerations alone, it will be appreciated that it is possible to achieve substantially 100% loading efficiency, i.e., loading of substantially all of the compound present into the liposomes, by proper selection of the initial external concentration of compound in relation to the known inside/outside volume ratio of the liposomes, which can be estimated.

Detailed Description Text (61):
In some cases, the weak acid compound, in associated form, is readily able to permeate the liposome transmembrane only at a temperature above the phase transition temperature of the liposomes. In such cases, loading of the compound into the liposomes is carried out at a temperature above the liposome phase transition temperature, and entrapment of the compound is effected by cooling the liposome suspension containing encapsulated compound below the lipid phase transition temperature. Entrapment carried out as described above offers prolonged stability to the encapsulated compound, particularly against degradative processes which might otherwise impact the compound in non-encapsulated form.

Detailed Description Text (63):
Liposomes having a higher inside/lower outside pH gradient in response to a transmembrane difference in acetate ion concentration are prepared as described above. Typically, weak acid compounds to be loaded are added to the bulk medium at concentrations ranging from 1 .mu.M-100 mM, with the concentration selected depending upon both the absolute quantity of drug intended for encapsulation and the degree of loading efficiency desired, as discussed above.

Detailed Description Text (64):
After adding the weak acid compound to the liposomes, the liposomes are treated under conditions effective to trap the compound within the liposomes. Conditions suitable for compound loading are those which (i) allow diffusion of the weak acid compound, with such in an uncharged form, into the liposomes, (ii) lead to a desired final loading concentration and efficiency, and (iii) provide a self-sustaining pH gradient after drug loading.

Detailed Description Text (65):
Considering the first of these requirements, the loading period may range from 1 minute to several hours, and is typically between 15-120 minutes, depending on permeability of the weak acid drug into the liposomes, temperature, and the relative concentrations of liposome lipid and drug. Where the compound is one which readily permeates the liposome membrane only above the phase transition temperature of the liposome lipids, e.g., 50.degree. C., the loading is carried out above this temperature. After loading, the liposomes are cooled below the phase transition temperature, e.g., to a storage temperature between 4-24.degree. C., such cooling acting to retard efflux of the loaded compound from the liposomes, independent of a pH gradient mechanism.

Detailed Description Text (66):
The final drug loading concentration and loading efficiency may be approximated from the Henderson-Hasselbach relationship, as discussed above. In addition to the considerations already discussed, the concentration of weak acid remaining after drug loading must be sufficient to maintain a high inside/low outside concentration gradient of the weak acid, preferably a ratio of at least 10:1. Thus, for example, if the initial concentration of weak acid is 150 mM, and the final concentration of loaded weak-acid compound is 50 mM, the final concentration of weak acid in the liposomes would be 100 mM (ignoring the loss of weak acid used in establishing the pH gradient), since capture of each molecule of compound within the liposomes, by deprotonating the compound, requires the shuttling of one proton out of the liposomes, and thus the efflux of one molecule of the weak acid from the liposomes. Assuming a V.sub.o /V.sub.i ratio of 50, concentration gradient of weak acid after drug loading in this example would be 100:50/50, or 100:1, sufficient to maintain a inside to outside drug loading ratio of 100:1.

Detailed Description Text (67):
In addition, the excess weak acid in the liposomes after drug loading provides a reservoir for sustaining the pH gradient across the liposomes over an extended storage time, as the equilibrium between protonated and unprotonated forms of the encapsulated weak acid is shifted in response to hydroxyl ion efflux or proton influx over time, as described in Section II. Accordingly, drug efflux from the liposomes on storage is effectively uncoupled from proton influx or hydroxyl-ion efflux, allowing for stable compound storage in suspension form over an extended period.

Detailed Description Text (69):
To illustrate one embodiment of the present invention, two exemplary weak acids were loaded into pH gradient liposomes. The compounds were loaded using as a driving force the pH gradient generated by a transmembrane difference in acetate concentrations, as described in Example 3. The model compounds selected for loading, 5(6)-carboxyfluorescein and nalidixic acid, are both fluorescent weak acid

compounds. Their fluorescent properties provided a useful means for determining the concentration of the compounds in liposomal media.

Detailed Description Text (80):
However, with proper selection of liposome concentration, external concentration of added compound, and the pH gradient, essentially all of the weak acid compound may be loaded into the liposomes. For example, with a pH gradient of 2-3 units (or the potential of such a gradient employing an acetate ion gradient), the final internal:external concentration of drug will be about 1000:1. Knowing the calculated internal liposome volume, and the maximum concentration of loaded drug, one can then select an amount of drug in the external medium which leads to substantially complete loading into the liposomes.

Detailed Description Text (85):
The experiment was carried out at two different temperatures to investigate how changes in lipid phase (i.e., gel versus liquid-crystalline) affects the penetration of molecules into the bilayer. When the suspension was incubated at 25.degree. C. (i.e., a temperature below the lipid phase transition temperature), addition of the ionophore at t=700 seconds had no effect on the fluorescence, as shown in FIG. 8 (solid line).

Detailed Description Text (86):
However, upon increasing the incubation temperature from 25.degree. C. to 60.degree. C., a significant effect on both the loading and the behavior of the ionophore was observed. Increasing the temperature above the lipid phase transition temperature during addition of the calcium ionophore resulted in a loading rate enhanced by one order of magnitude (t.sub.1/2 =84 s at 25.degree. C. versus 7 s at 60.degree. C.).

Detailed Description Text (101):
In the most general embodiment, the retaining means includes the weak-acid transmembrane gradient which is due to an excess of weak acid species in the liposomes after drug loading, and which provides a reservoir for sustaining the pH gradient, as discussed above.

Detailed Description Text (102):
Where the loaded compound is one which does not readily permeate the liposome membranes below the lipid phase transition temperature of the liposomes, the retaining means may additionally include the low-permeability barrier provided by the lipid bilayer.

Detailed Description Text (103):
Finally, where the loaded compound is one which has a low solubility in the presence of a selected cation, the cation itself provides retaining means by holding loading compound in a precipitated form that prevents efflux from the liposomes. In particular, it will be appreciated that the precipitating mechanism allows higher amounts of compound to be loaded stably into liposomes than is possible by a pH gradient alone, since the Henderson-Hasselbach relationship applies only to the solute form of the compound. For example, if the loaded compound precipitated above 5 mM compound concentration in the liposomes, a gradient effective to load to just above this relatively low concentration would be effective to load the liposomes to a high total compound concentration, e.g., 100-200 mM.

Detailed Description Text (107):
It will be appreciated how the features of the invention contribute to its applications in drug-delivery or other uses of compound-loaded liposomes. The weak-acid gradient liposomes used for compound loading are effective to generate their own pH gradient, and self-sustain this gradient by the shifting equilibrium between protonated and non-protonated forms of the encapsulated weak acid.

Detailed Description Text (109):
The liposomes, once loaded, are capable of retaining the compound at high concentration over an extended storage and/or drug-delivery period, by virtue of the self-sustaining gradient mechanism provided by the reservoir of weak acid in the liposomes. When coupled with other retaining means, including the use of high phase transition lipids and/or low compound solubility in the presence of the weak acid counterion, stable compound loading for periods of up to several months in suspension form may be achieved.

Detailed Description Text (118):
The resulting lipid mixture was hydrated at 70.degree. C. (a temperature above the lipid "gel" state, i.e., to the liquid-crystalline or fluid phase transition temperature) to form multilamellar vesicles (MLVs). The volume of the hydration medium was adjusted to obtain a 10% (w/v) lipid concentration. The lipid suspension was frozen in liquid N.sub.2, followed by thawing in a water bath maintained at 70.degree. C. The freeze-thaw cycle was repeated five times.

Detailed Description Text (133):
Two weak acid compounds, 5(6)-carboxyfluorescein and nalidixic acid, were selected for remote loading into pH gradient liposomes. Properties of the weak acids are given in Table II below.

Current US Original Classification (1):
424/450

Other Reference Publication (10):
Deamer, D., et al. "The Response of Fluorescent Amines to ph-Gradients Across Liposome Membranes," Biochem et Biophysica Acta 274: 323-335 (1972).

Other Reference Publication (14):
Lasic, D., et al., "Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery," Biochim. Biophys. Acta 1239: 145-156 (1995).

Other Reference Publication (16):
Nichols, J., et al., "Catecholamine Update and Concentration By Liposomes Maintaining pH Gradients," Biochim. Biophys. Acta 455: 269-271 (1976).

CLAIMS:

1. A method of forming liposomes having a higher inside/lower outside pH gradient, comprising:

preparing a suspension of liposomes in an aqueous solution of a weak acid salt comprising (i) an anion, which, in protonated form, is uncharged and is capable of freely permeating the transmembrane barrier of liposomes, and (ii) a counterion that is substantially lipid membrane impermeable,

adjusting the concentration of weak acid salt present in the external medium to produce a higher inside/lower outside concentration gradient of the weak acid salt, and

allowing the weak acid to distribute itself between inner and outer liposome compartments, with the weak acid acting as an inside-to-outside proton shuttle, thereby generating a higher inside/lower outside pH gradient.

6. A method of loading a weak-acid compound into liposomes, comprising:

adding the compound to a suspension of liposomes having a higher inside/lower outside gradient of a weak acid salt comprising (a) an anion, which, in protonated

form is uncharged and is capable of readily permeating the transmembrane barrier of the liposomes, and (b) a counterion that is substantially lipid membrane impermeable, wherein the weak acid acts as an inside-to-outside proton shuttle to generate a higher inside/lower outside pH gradient and an accumulation of the compound within the liposomes, and

by said adding, achieving uptake of the compound within the liposomes.

11. The method of claim 6, wherein (i) the compound in protonated form is readily able to permeate the liposome transmembrane barrier only at a temperature above the phase transition temperature of the liposomes, (ii) the compound and suspension are maintained at a temperature above the phase transition temperature during compound accumulation into the liposomes, and (iii) said method further comprises cooling the compound and suspension below such transition temperature after compound loading into the liposomes.

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L4: Entry 85 of 123 File: USPT Apr 18, 2000

DOCUMENT-IDENTIFIER: US 6051251 A
TITLE: Liposome loading method using a boronic acid compound

Brief Summary Text (10):
In the case of ionizable hydrophilic or amphipathic drugs, even greater drug-loading efficiency can be achieved by loading the drug into liposomes against a transmembrane ion gradient (Nichols, J. W., et al., Biochim. Biophys. Acta 455:269-271 (1976); Cramer, J., et al., Biochemical and Biophysical Research Communications 75 (2): 295-301 (1977)). This loading method, generally referred to as remote loading, typically involves a drug having an ionizable amine group which is loaded by adding it to a suspension of liposomes prepared to have a lower inside/higher outside ion gradient, often a pH gradient.

Detailed Description Text (11):
Lipids for use in the present invention may be relatively "fluid" lipids, meaning that the lipid phase has a relatively low lipid melting temperature, e.g., at or below room temperature, or alternately, relatively "rigid" lipids, meaning that the lipid has a relatively high melting point, e.g., at temperatures up to 50.degree. C. As a general rule, the more rigid, i.e., saturated lipids, contribute to greater membrane rigidity in the lipid bilayer structure, and thus to more stable drug retention after active drug loading. Preferred lipids of this type are those having phase transition temperatures above about 37.degree. C.

Detailed Description Text (22):
In one embodiment of the invention, the liposomes are prepared to include an ion gradient, such as a pH gradient or an amine gradient, across the liposome lipid bilayer. One method for preparing ion gradient liposomes is set forth in Example 1, where a mixture of liposome-forming lipids is dissolved in a suitable organic solvent and evaporated in a vessel to form a thin film. The film is then covered with an aqueous medium containing the solute species that will form the aqueous phase in the liposome interior spaces in the final liposome preparation. The lipid film hydrates to form multi-lamellar vesicles (MLVs), typically with heterogeneous sizes between about 0.1 to 10 microns. The liposome are then sized, as described above, to a uniform selected size range.

Detailed Description Text (25):
In the simplest approach for generating a pH gradient, the hydrated sized liposomes have a selected internal-medium pH. The suspension of the liposomes is titrated until a desired final pH is reached, or treated as above to exchange the external phase buffer with one having the desired external pH. For example, the original medium may have a pH of 5.5, in a selected buffer, e.g., glutamate or phosphate buffer, and the final external medium may have a pH of 8.5 in the same or different buffer. The internal and external media are preferably selected to contain about the same osmolarity, e.g., by suitable adjustment of the concentration of buffer, salt, or low molecular weight solute, such as sucrose.

Detailed Description Text (37):
Liposomes formed as described above are used in the method of the invention for loading a diol-containing compound. In accordance with the invention, the compound is added to a suspension of liposomes and the suspension is incubated under

conditions effective to allow passage of the compound from the external medium into the liposomes. Incubation conditions suitable for drug loading are those which (i) allow diffusion of the compound-boronic acid compound complex into the liposomes, and (ii) preferably lead to high drug loading concentration, e.g., 50-200 mM drug encapsulated. It will be appreciated that the incubation conditions, e.g., time and temperature, will vary according to the liposome composition and the therapeutic compound. In some cases, the liposome suspension is incubated with the compound and the boronic acid compound at a temperature above the phase transition temperature of the liposome lipids. Thus, for liposomes formed predominantly of saturated phospholipids, the loading temperature may be as high as 60.degree. C. or more. The loading time is typically between 15-120 minutes, depending on permeability of the complex formed between the therapeutic compound and the boronic acid compound.

Detailed Description Text (40):
In another embodiment of the invention, an ion gradient is used in conjunction with the boronic acid shuttle system. The use of an ion gradient results in a higher concentration of drug inside the liposomes. Several ion gradients, such as ammonium ion gradients or pH gradients can be used for therapeutic compounds containing ionizable amine groups.

Current US Original Classification (1):
424/450

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L4: Entry 83 of 123 File: USPT Jul 4, 2000

DOCUMENT-IDENTIFIER: US 6083530 A
TITLE: High drug:lipid formulations of liposomal-antineoplastic agents

Abstract Text (1):
A method for encapsulation of antineoplastic agents in liposomes is provided, having preferably a high drug:lipid ratio. Liposomes may be made by a process that loads the drug by an active mechanism using a transmembrane ion gradient, preferably a transmembrane pH gradient. Using this technique, trapping efficiencies approach 100%, and liposomes may be loaded with drug immediately prior to use, eliminating stability problems related to drug retention in the liposomes. Drug:lipid ratios employed are about 3-80 fold higher than for traditional liposome preparations, and the release rate of the drug from the liposomes is reduced. An assay method to determine free antineoplastic agents in a liposome preparation is also disclosed.

Brief Summary Text (15):
Mayer et al. found that the problems associated with efficient liposomal entrapment of the antineoplastic agent can be alleviated by employing transmembrane ion gradients (see PCT application 86/01102, published Feb. 27, 1986). Aside from inducing doxorubicin uptake, such transmembrane gradients also act to increase drug retention in the liposomes. The present invention discloses improved buffer compositions employed for the purposes of efficiently loading liposomes utilizing transmembrane ion, specifically, transmembrane pH gradients, and retaining the entrapped agent.

Brief Summary Text (19):
The present invention discloses an encapsulation procedure employing transmembrane pH gradients, which surmounts the demands related to both optimization of effect and pharmaceutical problems, and a drug to lipid weight ratio formulation which reduces the toxicity of the drug. The resulting liposome-antineoplastic agent formulation is very versatile in that the loading process is not limited to any particular lipid composition, liposome size, or charge. Inexpensive lipids can be employed, trapping efficiencies of about 100% for a wide range of lipid compositions and vesicle sizes are readily achieved, drug to lipid weight ration of greater than about 0.1:1 to about 3.0:1, which are higher than for previous formulations are achieved (thereby decreasing the lipid load),

Brief Summary Text (23):
The present invention discloses a liposome composition that comprises an antineoplastic agent and a lipid preferably a phospholipid, such as EPC and cholesterol, and wherein the liposomes have a transmembrane ion gradient preferably a pH gradient. The liposomes have a drug (antineoplastic agent) to lipid ratio of about greater than about 0.1:1 to about 3:1, most preferably about 0.3:1 to 3:1. The liposomes contain a release-inhibiting buffer combination such as citric acid/sodium carbonate, citric acid/sodium bis phosphate, or sodium carbonate/potassium sulfate-HEPES. The antineoplastic agent can be for example, an anthracycline such as doxorubicin, daunorubicin, or epirubicin, a vinca alkaloid such as vinblastine, or vincristine, a purine or pyrimidine derivative such as 5-fluorouracil, an alkylating agent such as mitoxanthrone, mechlorethamine hydrochloride or cyclophosphamide, or an antineoplastic antibiotic such as

mitomycin or bleomycin. The liposomes may comprise phospholipid such as egg phosphatidylcholine ("EPC"), hydrogenated soy phosphatidylcholine ("HSPC"), distearoylphosphatidylcholine ("DSPC"), dimyriatoylphosphatidylcholine ("DMPC"), dipalmitoylphosphatidylcholine, ("DPPC") or diarachidonoylphosphatidylcholine ("DAPC"), and may additionally comprise cholesterol, for example, in about a 55:45 phospholipid:cholesterol mol ratio. The liposomes may additionally comprise alpha tocopherol. The liposomes can be about 30 nm to about 2 microns in size, preferably about 100 to about 300 nm in diameter; large unilamellar vesicles. They can contain about 50 to 200 mg/ml lipid, more preferably about 90 to about 110 mg/ml lipid. The entrapment of the antineoplastic agent in the liposomes is from about 50 to about 100%, preferably about 90% to about 100%, more preferably about 98 to about 100%. These liposomes may be large unilamellar vesicles, and may be homogeneous or unimodal with regard to size distribution. The liposomes may be administered intravenously in a patient. Pharmaceutical preparations containing the antineoplastic agents entrapped in-the liposomes and pharmaceutically acceptable carriers or diluents are another embodiment of the present invention. The liposome compositions of the invention may be used to treat or stabilize a neoplastic disease, or prophylactically to prevent the onset or recurrence of a neoplastic disease. The composition of the present invention is, for example, provided as a three-component system. Where the antineoplastic agent is doxorubicin, the three component system comprises empty liposomes in an acidic solution of about pH 4.0, a basic solution, and the antineoplastic agent. The acidic solution is acetic acid buffer, oxalic acid buffer, or succinic acid buffer, preferably aqueous citric acid buffer. The basic solution is preferably sodium carbonate. The drug to lipid weight ratio is greater than about 0.1:1 to about 3:1.

Brief Summary Text (24):
The liposome compositions may be prepared by first forming the liposomes in a first aqueous medium, preferably a buffer, then acidifying or alkalinizing the medium, thereby establishing a pH gradient. The resulting acidified or alkalinized liposomes are then admixed with the antineoplastic agent, such as doxorubicin.

Brief Summary Text (25):
The liposomes of the invention may be dehydrated, either prior to or following the establishment of the transmembrane pH gradient. The liposomes may be large unilamellar vesicles, and may be comprised of long chain saturated lipids. In another aspect of the invention, a method for determining free antineoplastic agent in a liposome preparation (an assay method) is disclosed. For example, for doxorubicin, this method involves measuring an absorbance differential, preferably at about 600 nm before and after alkalinizing and solubilizing the liposomes of the preparation. More specifically, the absorbance of the doxorubicin-containing liposomes is measured at about 600 nM. The liposome preparation is then alkalinized and the absorbance is measured again at 600 n. The liposomes are then solubilized and the absorbance is again measured at 600 nm. The alkalinized liposomes are then compared to a color chart from which the percent of encapsulated agent may be determined.

Detailed Description Text (2):
The present invention demonstrates the efficient trapping of antineoplastic agents in liposomes exhibiting a transmembrane pH gradient which can result in a drug to lipid ratio significantly higher than previous liposomal systems. Also, liposomes of the formulations disclosed demonstrate a reduced rate of drug release. The invention involves liposomal formulations for use as drug carrier systems that entrap drugs such as the antineoplastic agents doxorubicin, vincristine, and 5-fluorouracil. These systems can be used to decrease the toxic effects of the antineoplastic agents employed.

Detailed Description Text (4):
As discussed above, the liposomes of the invention may be formed by any of the methods known, but preferably they are formed according to the procedures disclosed

in Bally et al., PCT Application No. 86/01102, published Feb. 27, 1986. This technique allows the loading of liposomes with ionizable antineoplastic agents to achieve interior concentrations considerably greater than the drugs solubility in aqueous solution at neutral pH and/or concentrations greater than can be obtained by passive entrapment techniques. In this technique, a transmembrane ion (pH) gradient is created across the membranes of the liposomes and the antineoplastic agent is loaded into the liposomes by means of the pH gradient. The transmembrane pH gradient is generated by creating a concentration gradient for one or more charged species (e.g., Na.sup.+) Cl.sup.-, K.sup.+, Li.sup.+, OH.sup.-, and preferably H.sup.+) across the liposome membranes, and these ion gradients drive the uptake of ionizable bioactive agents (drugs) across the membranes. In the present invention, transmembrane H.sup.+ (pH) gradients are preferably employed.

Detailed Description Text (6):
Similarly, anionic antineoplastic agents may be loaded into liposomes having a basic interior. Such loading is in response to the basic pH gradient imposed by exchanging the original medium for a more acidic medium. In the case of loading 5-fluorouracil, for example, the first medium is preferably relatively basic, for example, an aqueous solution such as a buffer at about pH 6.8 to about 11.0, and most preferably about pH 9.6. For example, 300 mM sodium carbonate may be used at pH about 9.6. Other basic aqueous solutions such as sodium hydroxide or sodium bis phosphate may also be employed.

Detailed Description Text (8):
Once the liposomes have been sized to the appropriate size distribution, the external medium may be replaced, by changing the original external medium to a new external medium having a different concentration of the one or more charged species (e.g., H.sup.+ ions), for example, a relatively basic or relatively acidic medium. The replacement of the external medium can be accomplished by changing the external pH, for example, in the case of doxorubicin, daunorubicin, or epirubicin, by adding a basic solution such as preferably sodium carbonate, at about pH 11.0, or a pH sufficient to result in a final pH of about 7.5-8.3, most preferably pH 7.8. In the case of vincristine, sodium bis phosphate is preferably employed, at about pH 6.8 to about pH 7.2, preferably at pH 7.0, or at a pH sufficient to result in a final pH of about 7.1. Other basic solutions that may be employed include but are not limited to sodium bicarbonate, sodium bin phosphate, sodium hydroxide, or potassium phosphate. Such a procedure creates the concentration gradient. In the case of 5-fluorouracil, the external medium is changed to a relatively acidic medium for example, with buffer such as preferably potassium sulfate/150 mM HEPES, or H.sub.2 SO.sub.4, at pH about 6.5 to about 8.5, added in sufficient amount to make the preparation relatively acidic, preferably about pH 7.0. Other relatively acidic solutions that may be used for FU include but are not limited to HCl, H.sub.3 PO.sub.4, to a desired pH of about 7.0. Other methods that may be used to change the external medium are gel filtration; (e.g. using a Sephadex column which has been equilibrated with the new medium), centrifugation, dialysis, or related techniques. This transmembrane pH gradient will load the drug into the liposomes such that the free vesicle-associated drug ratios reflect or are greater than predicted by [H.sup.+].sub.in /[H.sup.+].sub.out ratios. An ion gradient remains across liposome membranes even after the loading has been completed.

Detailed Description Text (9):
In addition to loading a single antineoplastic agent, the pH gradient loading method can be used to load multiple antineoplastic agents, either simultaneously or sequentially. Also, the liposomes into which the ionizable antineoplastic agents are loaded may themselves be pre-loaded with other antineoplastic agents or other drugs using conventional passive encapsulation techniques (e.g., by incorporating the drug in the buffer from which the liposomes are made). Since the conventionally loaded materials need not be ionizable, this approach provides great flexibility in preparing liposome-encapsulated "drug cocktails" for use in cancer therapies. These "drug cocktails" may also comprise two or 1more populations of liposomes (which

entrap the same or different antineoplastic agents), comprise different lipid formulations, or comprise different vesicle sizes. Such cocktails may be administered in order to achieve greater therapeutic efficacy, safety, prolonged drug release or targeting.

Detailed Description Text (11):
Turning now to the aspects of the invention relating to reducing the rate of release of an ionizable antineoplastic agent or other ionizable biologically-active agent from liposomes, it has been surprisingly found that the transmembrane pH gradient may also markedly reduce the rate of release across the liposome membranes. Thus, the liposomes are extremely stable regarding release of their contents. The reduced rate of drug release is created by the liposome interior buffering capacity; that is, the concentrations on the inside and outside of the liposomes of a charged species such as H.sup.+ ions (e.g., a pH gradient). For example, high interior buffering capacities, which require a larger influx of cations (such as the antineoplastic agent) to decrease the pH gradient, will lead to longer retention times. Further, once the interior buffering capacity is exhausted, the release rate of the antineoplastic agent (e.g., doxorubicin) will be increased. Loading the liposomes with the drug requires adjusting the ionic concentration of the external medium of the liposomes to form a chemical potential across the liposome membrane. Where the ion is the hydrogen cation, such an adjustment may be made by changing the pH by adding a solution of relatively acidic or basic pH. As previously stated, the release rate of the bioactive agent is mediated by the buffer. Certain buffer combinations (internal aqueous medium/external aqueous medium) have been found to enhance to uptake and reduce the release of the liposome contents. For example, for the drugs doxorubicin, epirubicin, and daunorubicin, the buffer combinations found most suitable for the retention of liposomal contents are citric acid/sodium carbonate. In the case of vincristine, the buffer combination most suitable is citric acid/sodium bis phosphate. In the case of 5-FU, the preferred buffer combination is sodium carbonate/sodium hydroxide or sodium carbonate/potassium sulfate-HEPES.

Detailed Description Text (12):
Doxorubicin retention in EPC/cholesterol (55:45) vesicles exhibiting a pH gradient can be increased by employing citrate/carbonate buffer systems such that less than about 5% drug release is observed over 24 h at 37.degree. C. This vesicle-entrapped dozorubicin also appears stable to serum components; less than 5% doxorubicin is released over 24 hours for vesicles incubated at 37.degree. C. in 95% fresh human serum. In association assays, where dozorubicin was incubated with HEPES buffer at pH 7.5, and citrate buffers (sodium citrate) at pH ranging from about 4.0-7.5, citrate interacts with doxorubicin and precipitates, whereas HEPES buffer does not. Such a buffer combination, that is, citrate/carbonate, acts to reduce the rate of release of the drug from the liposomes. Other release-reducing buffer combinations can be used such an oxalic acid/potassium phosphate or succinic acid/sodium bicarbonate, with citric acid/sodium carbonate or citric acid/sodium bis phosphate preferred.

Detailed Description Text (14):
Other methods are suitable for mixing the drug, buffers and liposomes. For example, saline may first be used to suspend the drug, then added to the liposomes having the transmembrane pH gradient. Additionally, the drug may be added to the liposome concurrent with the adjusting of the pH thereby creating the gradient. Other methods of mixing may be required depending upon the antineoplastic agent and other pharmaceutical components present.

Detailed Description Text (15):
The transmembrane pH gradient loading method can be used with essentially any antineoplastic agent which can exist in an ionizable state when dissolved in an appropriate aqueous medium (e.g., organic compounds which include an amino group which can be protonated). Those agents may contain primary, secondary, tertiary or

quaternary amine groups, and a lipophilic group, and should not dissipate the pH gradient. The agent should be relatively lipophilic so that it will partition into the liposome membranes. Examples of some of the antineoplastic agents which can be loaded into liposomes by this method and therefore may be used in this invention include but are not limited to anthracyclines such as doxorubicin, daunorubicin, mitoxanthrone, and epirubicin, antineoplastic antibiotics such as mitomycin and bleomycin, vinca alkaloids such as vinblastine and vincristine, alkylating agents such as cyclophosphamide and mechlorethamine hydrochloride, and purine and pyrimidine derivatives such as 5-fluorouracil (see Goodman and Gilman, eds., The Pharmacological Basis of Therapeutics, 6th ed., MacMillan & Co., 1980, pages 1249-1314. This invention is not to be limited to those drugs currently available, but extends to others not yet developed or commercially available, and which can be loaded using the transmembrane pH gradients.

Detailed Description Text (16):
In order to determine whether an ionizable antineoplastic agent will load into liposomes in response to a transmembrane pH gradient, EPC-containing liposomes are made (about 1.0 mM EPC) with a 3H'-DPPC tracer and with a relatively acidic or basic internal medium such as 300 mM citric acid at about pH 4.0. These liposomes are extruded about 10 times according to the LUVET procedure through 2 100 nm filters, followed by adjustment of the external pH to a relatively basic or acidic pH, for example, sodium carbonate, at about pH 11.0. Following the formation of the pH gradient, the agent to be loaded, spiked with a radioactive isotope of the agent, is admixed with the liposomes to about 200 uM (per 1.0 mM lipid used). The liposomes are separated from free, unentrapped agent on G50-M Sephadex minicolumns at 500.times.g for 3 minutes into 13.times.100 mm tubes, and radioactivity counted in a scintillation counter. Uptake of the drug in nmoles per umole of lipid is then plotted over incubation time. One hundred percent of the available doxorubicin is taken up into liposomes under these conditions.

Detailed Description Text (17):
In the case of doxorubicin, commercially available forms, such as powdered, solid, and methylparaben-containing forms (Adriamycin R.D.F., Adria Laboratories, Inc., Columbus, Ohio) may be used in the invention. When the methylparaben-containing form is employed, an aqueous solution such as saline may be added to that form, thereby dissolving it, followed by the admixing of this suspension with the liposomes which have the transmembrane pH gradient across their bilayers. Such admixing at 60.degree. C. for about 10 minutes results in more than about 98% encapsulation of the doxorubicin.

Detailed Description Text (18):
Lipids which can be used in the liposome formulations of the present invention include phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), sphingomyelin (SPM), and the like, alone or in combination. The phospholipids can be synthetic or derived from natural sources such as egg or soy. The phospholipids dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) may also be used. In the preferred embodiments, egg phosphatidylcholine (EPC), and cholesterol are used in preferably a 55:45 mole ratio. In other embodiments, distearoylphosphatidyl choline (DSPC), dipalmitoylphosphatidylcholine (DPPC), or hydrogenated soy phosphatidyleholine (HSPC) may be used in a mole ratio of 55:45 with cholesterol. Dimyristolyphosphatidylcholine (DMPC) and diarachidonoyl phosphatidylcholine (DAPC) may similarly be used. Due to the elevated transition temperatures (T.sub.c) of lipids such as DSPC (T.sub.c of about 65.degree. C.), DPPC (T.sub.c of about 45.degree. C.), and DAPC (T.sub.c of about 85.degree. C.), such lipids are preferably heated to about their T.sub.c or temperatures slightly higher (e.g., up to about 5.degree. C. higher) than the T.sub.c in order to make these liposomes.

Detailed Description Text (42):

a transmembrane pH gradient where the interior of the liposomes is acidic, the first component of the system (Vial 1) is liposomes in an acidic solution, for example, in citric acid buffer (300 mmol., pH 3.8-4.2, preferably pH 4.0). The second component (Vial 2) is a base, preferably sodium carbonate or sodium bisphosphate solution at 0.5 M, pH 11.5. The third component (Vial 3) is the antineoplastic agent. The above-mentioned treatment system may be provided as a 3-vial system, with a first vial containing the liposomes in acidic medium, the second vial containing the base, and a third vial containing the antineoplastic agent (e.g. doxorubicin). Where the drug is one that loads in response to a transmembrane gradient wherein the inside of the liposomes is relatively basic (such an, for example, 5-FU), the first component of the system is liposomes in relatively basic buffer (such as, for example, sodium carbonate, pH 6.8-11.0, preferably pH 9.6). The second component is a relatively acidic solution, for example, 150 mM potassium sulfate/150 mM HEPES buffer, pH 7.4. The third component comprises the antineoplastic agent. Following the formation of the pH gradient across the liposomes (by admixing the first and second vials), the liposomes may be heated prior to admixing with the drug. When loading doxorubicin, vincristine, and FU it has been found advantageous to heat the liposomes to about 60.degree. C. Daunorubicin, epirubicin, mitoxanthrone, and vincristine load efficiently at 25.degree. C.

Detailed Description Text (44):
In the case of loading vincristine, the above protocol may similarly be employed, but the mixing sequence may be altered. For example, the vincristine may be admixed with the liposomes at acidic pH (pH 4.0), then the pH gradient established by the addition of a relatively basic solution.

Detailed Description Text (47):
The spectroscopic analysis of liposomal doxorubicin preparations was compared to column chromatography methods which directly measure free and vesicle associated drug to correlate absorbance ratio values to actual free DOX/total DOX ratios over a wide range of trapping efficiencies. Since pH gradients induce the uptake of doxorubicin into liposomes such that [DOX].sub.in /[DOX].sub.out ratios reflect [H.sup.+].sub.in /[H.sup.+].sub.out ratios, EPC/cholesterol liposomes exhibiting pH gradients (acidic inside) of varying magnitude were utilized to construct liposome systems with trapping efficiencies from 10 to 99%. FIG. 5 demonstrates that the absorbance ratio at 600 nm described here accurately represents the ratio of free/total doxorubicin in the vesicle preparations over the full range of trapping efficiencies studied. The spectroscopic analysis method was also completed on EPC liposomes in which doxorubicin had been passively entrapped during vesicle formation to insure that these results were not specific to liposomal doxorubicin obtained by active entrapment. FIG. 5 (open symbol) shows that the absorbance ratio at 600 nm for this sample correlates with the free/total doxorubicin value obtained by column chromatography.

Detailed Description Text (56):
When the dehydrated liposomes are to be used, rehydration is accomplished by simply adding an aqueous solution, e.g., distilled water or an appropriate buffer, to the liposomes and allowing them to rehydrate. The liposomes can be resuspended into the aqueous solution by gentle swirling of the solution. The rehydration can be performed at room temperature or at other temperatures appropriate to the composition of the liposomes and their internal contents. If the antineoplastic agent which is to be administered was incorporated into the high drug to lipid ratio liposomes prior to dehydration, and no further composition changes are desired, the rehydrated liposomes can be used directly in the cancer therapy following known procedures for administering liposome encapsulated drugs. Alternatively, using the transmembrane pH gradient procedures described above, ionizable antineoplastic agents can be incorporated into the rehydrated liposomes just prior to administration. In connection with this approach, the concentration gradient used to generate the transmembrane pH gradient can be created either

before dehydration or after rehydration using the external medium exchange techniques described above. For example, the high drug to lipid ratio liposomes may be dehydrated prior to establishing the transmembrane pH gradient, for example, dehydrated from their first external medium. Upon rehydration, the pH gradient can be established by admixing the liposomes with the second external medium of relatively acidic or basic pH. The antineoplastic agent can be admixed-with the liposomes simultaneously with or following the establishment of the pH gradient.

Detailed Description Text (57):
In the case where the liposomes are dehydrated after having a transmembrane pH gradient, the liposomes may be rehydrated by admixing them with an aqueous solution of neutral pH.

Detailed Description Text (58):
For example, in the above-mentioned case where liposomes containing citric acid buffer as the first external medium are used, the rehydration step would proceed by adding sodium carbonate and the antineoplastic agent, such as dozorubicin. Where the liposomes already containing the base (e.g. sodium carbonate), and therefore already have the transmembrane pH gradient are rehydrated, water or another neutral aqueous solution, and doxorubicin are added. Finally, in the case where liposomes having a transmembrane pH gradient and containing doxorubicin have been dehydrated, rehydration proceeds using water or another aqueous solution. Alternatively, another antineoplastic agent may be added, if desired.

Detailed Description Text (157):
The above liposomes containing FU were then passed down a Sephadex G-50 column equilibrated with 150 mM NaCl at 37.degree. C. 5-FU re-equilibrated according to the pH gradient.

Detailed Description Text (166):
The materials and procedures of Example 30 were employed except that cisplatin (200 uM) was combined with the liposome suspension. Cisplatin was not accumulated into liposomes by the transmembrane pH gradient.

Current US Original Classification (1):
424/450

Other Reference Publication (7):
Deamer, et al., "The response to Fluorescent Amine to pH Gradients Across Liposome Membranes", 1972, Biochim. Biophys, Acta, 274, p323-335.

Other Reference Publication (29):
Nichols and Deamer, "Catecholamine Uptake and Concentration by Liposomes Maintaining pH Gradients" 1976, BBA 455,p. 269-271.

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L4: Entry 82 of 123 File: USPT Aug 29, 2000

DOCUMENT-IDENTIFIER: US 6110491 A
TITLE: Compound-loaded liposomes and methods for their preparation

Brief Summary Text (25):
Compound loading against an inside-to-outside pH or electrochemical liposome gradient has proven useful for loading ionizable compounds into liposomes. In theory, very high loading efficiencies can be achieved by employing suitable gradients, e.g., pH gradients of 2-4 units, and by proper selection of initial loading conditions (Nichols and Deamer, 1976). With this method, compound leakage from the liposomes will follow the loss of ion gradient from the liposomes. Therefore, compound can be stably retained in liposome-encapsulated form only as long as the ion gradient is maintained.

Brief Summary Text (36):
The incubating step may be carried out at a temperature above the phase transition temperature of lipids forming the liposomes. The method may further include removing unprecipitated compound from the bulk phase of the suspension, after compound loading.

Brief Summary Text (39):
suspension of liposomes having an ionizable compound stably encapsulated in the liposomes in precipitated form, where the compound is relatively water insoluble at a first pH, and relatively water soluble at a second pH. The method includes adding the compound to a dispersion of liposomes contained in a bulk-phase aqueous medium, where the dispersion has an inside-to-outside liposome pH gradient corresponding to said first and second pH, respectively. The compound and dispersion are incubated under conditions which allow uptake of the compound by the liposomes to a compound concentration that is severalfold that of the compound concentration in the bulk-phase medium, as evidenced by the formation of a precipitate inside the liposomes.

Brief Summary Text (40):
In one embodiment, the inside-to-outside pH gradient may be opposite to that required for loading the same compound in soluble form into liposomes. The method may further include adjusting the pH of the bulk phase medium to that of the internal liposome pH following the incubating step.

Brief Summary Text (41):
In a final aspect, the invention includes a method of producing a suspension of liposomes having an ionizable compound stably encapsulated in the liposomes in precipitated form. The method includes adding the compound to a dispersion of liposomes contained in a bulk-phase aqueous medium, where the liposomes have (i) a higher inside/lower outside gradient of a multivalent, charged precipitating agent, and (ii) substantially no inside-to-outside pH gradient. The compound and dispersion are then incubated under conditions that allow uptake of the compound by the liposomes to a compound concentration that is severalfold that of the compound concentration in the bulk-phase medium, as evidenced by the formation of a precipitate inside the liposomes.

Detailed Description Text (40):
Liposomes having a more rigid lipid bilayer, or a liquid crystalline bilayer, are

achieved by incorporation of a relatively rigid lipid, e.g., a lipid having a relatively high phase transition temperature, e.g., up to 60.degree. C. Rigid, i.e., saturated, lipids contribute to greater membrane rigidity in the lipid bilayer. Other lipid components, such as cholesterol, are also known to contribute to membrane rigidity in lipid bilayer structures.

Detailed Description Text (41):
On the other hand, lipid fluidity is achieved by incorporation of a relatively fluid lipid, typically one having a lipid phase with a relatively low liquid to liquid-crystalline phase transition temperature, e.g., at or below room temperature.

Detailed Description Text (48):
Typically, the incubating is carried out at an elevated temperature, and preferably above the phase transition temperature T.sub.p of the liposome lipids. For high-phase transition lipids having a T.sub.p of 50.degree. C., for example, incubation may be carried out at between 55-60.degree. C. The incubation time may vary from between an hour or less to up to 12 hours or more, depending on incubation temperature and the rate of permeation of the compound through the liposome membrane.

Detailed Description Text (52):
In practicing the method, the compound is added to a dispersion of liposomes having an inside-to-outside liposome pH gradient corresponding to the first and second pH, respectively. Specifically, the internal liposome pH is one at which the compound precipitates at low compound concentration, and the bulk phase pH is one at which the compound is relatively soluble. Exemplary compound solubilities for a variety of compounds suitable in the invention are given in Section IB above.

Detailed Description Text (59):
In practicing the method, there is first prepared a dispersion of liposomes contained in a bulk-phase aqueous medium, and having (i) a higher inside/lower outside gradient of a multivalent, charged precipitating agent, and (ii) substantially no inside-to-outside pH gradient.

Detailed Description Text (84):
The liposomes prepared according to the present invention can be clearly distinguished from similar liposomes prepared by pH gradient, or ammonium ion gradient methods, by the liposomal response to membrane-active ion carriers which destroy transmembrane electrochemical gradients. For example, CCCP is a membrane proton carrier which destroys transmembrane pH gradients and leads to quick release of a liposomal-entrapped compound. Nigericin, a membrane carrier for protons and monovalent ions, quickly releases doxorubicin from liposomes loaded by the ammonium ion method (Haran, et al., 1993).

Detailed Description Text (88):
Liposomes with entrapped ammonium sulfate or ammonium polyacrylate were prepared from the lipid mixture of hydrogenated soybean phosphatidylcholine (Avanti PolarLipids, Ala., U.S.A.), cholesterol (Calbiochem, USA), and poly(ethylene glycol) (Mol. weight 2,000) derivative of distearoyl phosphatidyl ethanolamine (PEG-DSPE) (Sygena, Switzerland), at the molar ratio 60:40:6, by lipid film hydration, repetitive freezing-thawing at 60.degree. C. (6 times) and extrusion through two stacked polycarbonate track-etched membranes with the pore size 100 nm at 60.degree. C. (12 times). The bulk aqueous phase of the liposomes was exchanged by gel-filtration for the outer buffer, 0.2 M ammonium chloride, adjusted (when necessary) to pH 7.3 and buffered with 10 mM sodium hydroxyethylpiperazino-ethane sulfonate (HEPES). The inner (entrapped) solution had ammonium ion concentration of 200 milli-eqivalent/L, i.e. the same as the outer buffer (no ammonium ion gradient), and the anion composition and pH as indicated below. Polyacrylic acid with mol. weight of 2,000 (Aldrich Chemical Co.) was used. Doxorubicin was added to

the liposomes at 2 mg for each 6-8 micro-mol of phospholipid, and incubated with shaking at 60.degree. C., i.e. above the transition temperature of the lipid bilayer, for various times as specified below. At this temperature, doxorubicin did not form a detectable precipitate in the presence of sulfate anion, but was visibly precipitated by a polyacrylate anion. Doxorubicin-loaded liposomes were separated from the free drug by gel-chromatography on Sephadex G-75, eluted with the outer buffer. Liposomal doxorubicin was assayed by spectrophotometry at 485 nm, phospholipid was quantitated by molybdate method after acid digestion of the liposomes. The following results were obtained:

Detailed Description Text (92):
Aqueous solutions containing ammonium salts of hydrochloric, nitric, methanesulfonic, L(+)-tartaric, citric, sulfuric, phosphoric, diethylenetriamine pentaacetic (DTPA), or polyacrylic (Mol. weight. 2,000) acids were prepared by titration of the respective acids in water with the standard aqueous solution of ammonium hydroxide to achieve pH 7.3.+-.0.05, followed by volume adjustment to achieve 0.2 equivalent/L of ammonium ion, and, if necessary, by addition of dry sucrose to achieve osmolarity of 377 mmol/kg. (These solutions are hereinafter referred to as Inner Buffers). All solutions additionally contained 10 mM hydroxyethylpiperazinoethane sulfonic acid (HEPES) to stabilize the pH at titration endpoint. To afford determination of intraliposomal pH, a fluorescence indicator 8-hydroxypyrene trisulfonate was added to the solutions to the final concentration of 0.2 mM. Liposomes containing entrapped ammonium salt solutions as above were prepared from egg phosphatidyl choline, cholesterol, and PEG-DSPE as described in the Example 7, except that lipid hydration and extrusion were carried out at room temperature. The bulk aqueous phases of liposome preparations were exchanged by gel-filtration for an aqueous solution (Outer buffer) containing 0.2 M ammonium chloride, 10 mM HEPES, pH 7.3, osmolarity 377 mmol/kg, to obtain liposome preparations substantially without transmembrane ammonium-ion gradients. Loading of doxorubicin was performed as in Example 7, except that it was at 36.degree. C. for 12 hours. Intraliposomal pH was determined using HPTS fluorescence method (Straubinger, et al., 1990) prior to drug loading, and indicated essentially no inside-outside pH gradient (less than 0.2 pH units), compared with the gradient of several pH units typical for ammonium ion gradient liposomes (Haran, et al., 1993). The following results were obtained (nd, not determined)

Detailed Description Paragraph Table (7):
_____ Doxorubicin incorporation pH gradient, into liposomes, mg of Inner Buffer anion inside vs. drug/mmol of liposome composition outside. phospholipid _____ chloride -0.11 16.8 nitrate -0.16 12.4 methanesulfonate -0.21 17.1 L(+)-tartrate nd 27.5 sulfate nd 33.5 DTPA nd 40.8 phosphate nd 68.1 citrate -0.13 97.9 polyacrylate -0.02 142.7

Current US Original Classification (1):
424/450

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L4: Entry 80 of 123 File: USPT Mar 13, 2001

DOCUMENT-IDENTIFIER: US 6200598 B1
** See image for Certificate of Correction **
TITLE: Temperature-sensitive liposomal formulation

Abstract Text (1):
Temperature sensitive liposomes containing active agents are described. The liposomes comprise phospholipids and a surface active agent, such as a lysolipid, that increases the amount of active agent released by the liposome at the phase transition temperature of the liposome. Such liposomes are useful in methods of administering active agents using mild hyperthermic conditions.

Brief Summary Text (7):
Liposomes are normally not leaky but will become so if a hole occurs in the liposome membrane, if the membrane degrades or dissolves, or if the membrane temperature is increased to the phase transition temperature. The elevation of temperature (hyperthermia) at a target site in a subject to raise liposome temperature above the phase transition temperature, and thereby cause the release of the liposome contents, has been used for the selective delivery of therapeutic agents. Yatvin et al., Science 204:188 (1979). This technique is limited, however, where the phase transition temperature of the liposome is significantly higher than the normal tissue temperature.

Brief Summary Text (10):
In view of the foregoing, a first aspect of the present invention is a liposome containing an active agent. The liposome's lipid bilayer membrane contains phospholipids as the primary lipid source; lysolipids are contained in the bilayer membrane in an amount that increases the percentage of active agent released at the phase transition temperature, compared to that which would occur in the absence of lysolipid.

Brief Summary Text (14):
A further aspect of the present invention is a method of making liposomes containing an active agent in the liposome interior space. A phospholipid film containing lysolipid is first prepared, and then hydrated with an aqueous preparation containing the active agent and an equilibrating amount of lysolipid monomer. Lysolipid is contained in the liposome membrane in an amount sufficient to increase the percentage of active agent released at the phase transition temperature of the liposome membrane, compared to that which would occur in the absence of the lysolipid.

Drawing Description Text (6):
FIG. 5A provides heat flow thermograms showing the effect of varied MPPC concentration on the phase transition temperature (Tc) of DPPC liposomes. The Tc of lyophilized liposomal samples of DPPC containing MPPC (1-10 mol %) was measured by Differential Scanning Calorimetry (DSC) between 30.degree. C.-45.degree. C. with 2 C.degree./minute heating rate.

Drawing Description Text (7):
FIG. 5B graphs the effect of MPPC concentration on the phase transition temperature (Tc) of DPPC liposomes, as described above for FIG. 5A. The graph shows the start

point of the transition (open circle); the peak in enthalpy (closed circle); and the end point of the transition (open triangle).

Detailed Description Text (2):
The present invention provides liposomes that are sensitive to alterations in the temperature of the surrounding environment. The temperature-sensitivity of such liposomes allows the release of compounds entrapped within the interior aqueous space of the liposome, and/or the release of compounds associated with the lipid bilayer, at a target site that is either heated (as in the clinical procedure of hyperthermia) or that is at an intrinsically higher temperature than the rest of the body (as in inflammation). Liposome bilayers of the present invention include (in addition to a primary or main lipid component) lysolipid, or another surface active agent(s). The inclusion of lysolipid or another surface active agent in the liposome bilayer enhances the release of compounds when the liposome temperature reaches the gel-to-liquid crystalline phase transition temperature of the primary lipid component. The presence of the lysolipid (or other surface active agent) also causes the liposome to release the drug at a slightly lower temperature than that achieved with liposomes composed solely of phospholipids. Liposomes of the present invention are particularly useful in drug delivery, where the liposome contains a compound to be delivered to a preselected target site in a subject's body. The target site is either artificially heated (hyperthermia) so that it is at or above the gel-to-liquid crystalline phase transition temperature, or the target site may be at a higher temperature than non-targeted sites in the body due to natural causes (e.g., inflammation), where that temperature is at or above the gel-to-liquid crystalline phase transition temperature of the liposome utilized.

Detailed Description Text (3):
When liposomes are incubated for several minutes at temperatures in the region of the gel-to-liquid crystalline phase transition temperature (Tc) of the primary lipid composing the liposome, the liposome bilayer becomes permeable and releases solutes entrapped within the liposome into the surrounding solution. The clinical use of hyperthermia with such thermally-sensitive liposomes has been proposed. See, e.g., Yatvin et al., Science 202:1290 (1978).

Detailed Description Text (10):
Liposomes of the present invention comprise a lipid possessing a gel-to-liquid crystalline transition temperature in the hyperthermic range (e.g., the range of from approximately 38.degree. C. to approximately 45.degree. C.). Preferred are phospholipids with a phase-transition temperature of from about 38.degree. C. to about 45.degree. C., and more preferred are phospholipids whose acyl groups are saturated. A particularly preferred phospholipid is dipalmitoylphosphatidylcholine (DPPC). DPPC is a common saturated chain (C16) phospholipid with a bilayer transition of 41.5.degree. C. (Blume, Biochemistry 22:5436 (1983); Albon and Sturtevant, Proc. Natl. Acad. Sci. USA 75:2258 (1978)). Thermosensitive liposomes containing DPPC and other lipids that have a similar or higher transition temperature, and that mix ideally with DPPC (such 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (DPPG) (Tc=41.5.degree. C.) and 1,2-Distearoyl-sn-Glycero-3-Phosphocholine (DSPC) (Tc=55.1.degree. C.)) have been studied. Kastumi Iga et al, Intl. J. Pharmaceutics, 57:241 (1989); Bassett et al, J. Urology, 135:612 (1985); Gaber et al, Pharmacol. Res. 12:1407 (1995). Thermosensitive liposomes containing DPPC and cholesterol have also been described. Demel and De Kruyff, Biochim. Biophys. Acta. 457:109 (1976).

Detailed Description Text (12):
Liposomes of the present invention incorporate a relatively-water soluble surface active agent, such as a lysolipid, into a bilayer composed primarily of a relatively water-insoluble molecule, such as a di-chain phospholipid (e.g., DPPC). Incorporation of the surface active agent in the gel phase of the primary lipid component enhances the release of contents from the resulting liposome when heated to the gel-liquid crystalline phase transition temperature of the primary lipid.

Preferred surface active agents are lysolipids, and a particularly preferred surface active agent is monopalmitoylphosphatidylcholine (MPPC). Suitable surface-active agents are those that are compatible with the primary lipid of the bilayer, and that desorb when the lipid melts to the liquid phase. Additional suitable surface-active agents for use in phospholipid bilayers include palmitoyl alcohols, stearoyl alcohols, palmitoyl, stearoyl, polyethylene glycol, glyceryl monopalmitate, glyceryl monooleate, ceramides, PEG-ceramides, and therapeutic lipids. Therapeutic lipids include, for example, C-18 ether linked lysophoshpatidylchohline.

Detailed Description Text (14):
The present invention provides liposomes that release entrapped contents at temperatures that can be achieved in clinical settings using mild hyperthermia. The present invention provides liposomes (THERMOSOMES.TM.) that are highly stable at body temperature (37.degree. C.) but that become unstable and show enhanced release of entrapped compounds at temperatures beyond about 39.degree. C. This temperature range is a few degrees below that of many previous liposomal formulations that only showed significant release at temperatures greater than 42.degree. C. Additionally, the present liposomal formulation's combination of lipid and compatible lysolipid provides a lipid/lysolipid mixture with a slightly lower gel-to-liquid crystalline transition temperature compared to that of pure lipid alone, yet the gel-to-liquid crystalline transition temperature is not broadened by the inclusion of a lysolipid.

Detailed Description Text (15):
A preferred liposome of the present invention has a bilayer composed primarily of phospholipid, and containing lysolipid in an amount that decreases the gel-to-liquid crystalline phase transition temperature of the bilayer, compared to a bilayer composed of phospholipid alone. A particularly preferred liposome of the present invention comprises a DPPC as the primary phospholipid and MPPC as the lysolipid, where the ratio of DPPC:MPPC is from about 99:1, 98:2, 97:3, 96:4, 95:5, 90:10, to about 80:20, 75:25, 70:30, 65:35, 60:40, or even 51:49 (by molar ratio).

Detailed Description Text (17):
While not wishing to be held to any single theory of action, the present inventors believe that the mechanism whereby lysolipids (or other surface active agents) enhance the release of contents from liposomes composed primarily of phospholipid is related to the way in which the lysolipid is mostly ideally mixed in the mixed gel phase bilayer, but creates defects at the microstructural level (microdomain boundaries) as it desorbs from the membrane upon bilayer melting at the primary acyl chain transition temperature (i.e., at the transition temperature of the primary bilayer lipid). The inclusion of a surface active agent such as a lysolipid lowers the phase transition temperature of a phospholipid membrane, compared to the phase transition temperature of a membrane composed solely of the phospholipid. In a liposome composed of DPPC and MPPC, the phase transition temperature is lowered depending on the amount of MPPC incorporated into the gel phase bilayer; the reduction of phase transition temperature that can be achieved is limited by the amount of MPPC that can be stably contained in the bilayer. Membranes composed of DPPC can stably contain from 1 mol % MPPC, up to about 20 mole % MPPC, 30% mole % MPPC, 40 mol % MPPC, or even 50 mol % MPPC, depending on other conditions such as the active agent contained within the liposome.

Detailed Description Text (18):
In liposome bilayers containing phospholipid and a surface active agent such as a lysolipid, it is preferable that the surface active agent be contained in both layers of the bilayer. This concept is illustrated in FIG. 1, which schematically represents a liposome composed of DPPC and MPPC. The molecules of MPPC are present in both the exterior and the interior layer of the liposome membrane bilayer. In a liposome containing surface active agent in only one layer of the bilayer, redistribution of the surface active agent to both layers of the bilayer will occur

over time at temperatures above the gel transition temperature (e.g., in the liquid crystalline phase).

Detailed Description Text (19):
Phase compatibility of the two (or more) components of the present invention affects the processing and stability of the lipid bilayer structure. In liposomes composed primarily of DPPC (a di-chain phospholipid), to maximize compatibility and preserve the narrow melting range of the main phospholipid, a preferred lysolipid is MPPC because it is identical to the di-chain phospholipid except that it possesses only one acyl chain (FIGS. 11A and 11B). The present inventors discovered that inclusion of this bilayer-compatible lysolipid in low concentrations (preferably 2-20 mol %), makes liposomes composed primarily of DPPC more "leaky" at the point at which the primary lipid begins to melt (i.e., the solidus line of the main phase transition), compared to liposomes composed of DPPC alone. While not wishing to be held to a single explanation, the present inventors believe that gel phase bilayers are composed of microcrystalline domains; as the temperature approaches the gel-to-liquid crystalline phase transition of the lipid bilayer, membrane permeability to the entrapped drug increases at the grain boundaries of the microstructure. At the transition temperature, desorption of the lysolipid dissolved in the gel phase microstructure enhances the membrane permeability. An additional benefit of incorporating a compatible molecule in the liposome bilayer is that the phase transition temperature of the primary lipid is not broadened, but is lowered by about a degree or more (depending on the lysolipid concentration in the bilayer).

Detailed Description Text (23):
A method of preparing a liposomal formulation according to the present invention comprises mixing the bilayer components in the appropriate proportions in a suitable organic solvent, as is known in the art. The solvent is then evaporated to form a dried lipid film. The film is rehydrated (at temperatures above the phase transition temperature of the lipid mixture) using an aqueous solution containing an equilibrating amount of the surface active agent and a desired active agent. The liposomes formed after rehydration can be extruded to form liposomes of a desired size, as is known in the art. For example, where liposomes composed of 80:20 DPPC:MPPC are produced, rehydration is carried out at a temperature above the phase transition temperature of this particular lipid mixture (above 39.degree. C.). The aqueous solution used to rehydrate the lipid film comprises an equilibrating amount of lysolipid monomers (e.g., a concentration equal to the Critical Micelle Concentration of MPPC).

Detailed Description Text (28):
The incorporation of certain active agents (such as some anesthetics) in liposomes of the present invention may additionally alter (enhance or inhibit) the release of contents from the liposome, or alter the transition temperature of the liposome, compared to that which would be seen in a similar liposome that did not contain the active agent.

Detailed Description Text (37):
Liposomes of the present invention may be administered using methods that are known to those skilled in the art, including but not limited to delivery into the bloodstream of a subject or subcutaneous administration of liposomes. Where liposomes according to the present invention are used in conjunction with hyperthermia, the liposomes may be administered by any suitable means that results in delivery of the liposomes to the treatment site. For example, liposomes may be administered intravenously and thereby brought to the site of a tumor by the normal blood flow; heating of this site results in the liposomal membranes being heated to the phase transition temperature so that the liposomal contents are preferentially released at the site of the tumor.

Detailed Description Text (61):

FIG. 4 shows the release of CF from liposomes incubated for five minutes at temperatures of 20.degree. C. to 45.degree. C. in the presence of 10 mM PBS (pH=7.4). Release of CF was measured fluorimetrically at .lambda.ex=470 nm and .lambda.em=520 nm. The percent of CF released was calculated by comparing the values obtained with those obtained after the total release of CF (achieved by the addition of Triton X-100 to the liposome sample to dissolve the liposomes and release all entrapped CF). Pure DPPC liposomes were stable up to 39.5.degree. C. but became permeable near the transition temperature of the phospholipid, thus causing release of some of the CF. The amount of CF released from the pure DPPC liposome was, however, only about 20% of total contents. In contrast, with increasing concentrations of MPPC in the DPPC bilayers, liposomes showed an increasing release of CF, with maximum release occurring for the liposomes having bilayers containing 10 mol % and 20 mol % MPPC.

Detailed Description Text (65):
In DPPC liposomes containing MPPC concentrations of more than 20 mol %, the liposomes became intrinsically unstable and therefore unable to retain entrapped material at temperatures above the lipid phase transition (i.e., in the liquid phase of the lipid bilayer that occurs during processing of liposomes). Such high concentrations of MPPC can also destabilize the gel phase bilayers at temperatures below the transition temperature. As demonstrated previously, the mechanical strength of membranes decreases as more and more MPPC is included (Needham et al., Biophys. J. 73:2615 (997)), and the bilayers eventually make a transition to a pure micelle suspension as the mole ratio of MPPC to phospholipid goes beyond 50 mol % (Zhelev et al., Biophys. J. (in press; 1998)). One preferred molar ratio for thermally sensitive liposomes is 90:10, DPPC:MPPC.

Detailed Description Text (67):
Phase Transition Behavior of DPPC/MPPC Mixtures and Correlations with Release Temperatures

Detailed Description Text (68):
To investigate the biophysical mechanism involved in the permeability of the liposomes of the present invention, differential scanning calorimetric (DSC) studies were carried out to generate differential calorimetric thermograms for the present liposomes, to determine the phase transition temperature. These results were compared with the release versus temperature scans obtained from cumulative release profiles (shown in FIG. 4). FIGS. 5A and 5B show the heat flow thermograms for liposome preparations containing increasing concentrations of MPPC in DPPC bilayers. These thermograms show that the transition temperature remains unbroadened even though up to 10% of MPPC are included in the bilayer. At a higher level of resolution, FIG. 5B shows the change in the peak of the transition temperature from 41.9.degree. C. to 41.04.degree. C. as the MPPC composition is increased from zero to 10 mol % in DPPC bilayers. Also shown is the breadth of the transition, represented as the start and end point of the transition, i.e., the solidus and liquidus lines below and above the excess heat flow peak.

Detailed Description Text (69):
The differential scanning thermogram of liposomes of the present invention can be compared with the differential release profiles. FIG. 6A shows the cumulative release profile for CF release from the DPPC:MPPC 90:10 liposomes versus temperature, and the heat flow thermogram over the same temperature range. FIG. 6B shows the differential release, which highlights the sharpness of the release profile and the temperature at which maximum release occurs in relation to the heat flow thermogram. What is striking about this comparison is that that the peak release of contents obtained from the differential release profiles was 0.9.degree. C. lower than the peak in the transition enthalpy obtained by DSC. The release of the entrapped material at the temperature prior to Tc can be attributed to the fact that the release is occurring at the `solidus` line of the thermogram and not at the peak temperature. One explanation for such a behavior is that the release of

entrapped material occurs as soon as the `first defects` (melting defects) in the microdomain boundaries of the bilayer network appear; it is here that the lysolipid may exert its effects. While not wishing to be held to a single theory, the present inventors suspect that as the transition temperature is approached the first parts of the microstructure that melt are at the grain boundaries of the solid membrane. When surrounded by a lysolipid-free aqueous phase, the lysolipid is trapped in the gel phase but can desorb when the membrane begins to melt; as it does so it enhances the defect permeability and the contents are released more effectively than in liposomes of pure lipid alone.

Detailed Description Text (72):
Doxorubicin (DX) was entrapped into the inner aqueous volumes of liposomes of the present invention (DPPC:MPPC 90:10) using the pH gradient-driven encapsulation protocol (L. D. Mayer et al (1989) Cancer Res., 42:4734.). Briefly, a lipid composition of 90:10 DPPC:MPPC was dissolved in chloroform and the solvent was evaporated under vacuum at 45.degree. C. using a rotavapor. The lipid film obtained after further drying in the vacuum desiccator overnight was hydrated with 300 mM citrate buffer (pH 4.00) and the multilamellar vesicles formed were subjected to seven freeze-and-thaw cycles. The resulting suspension was extruded through two polycarbonate membrane filters of pore size 0.1 .mu.M at 50.degree. C. using 300-400 psi pressure. The extruded liposomes were allowed to cool to room temperature and the pH was raised to 7.5-8.0 using 0.5 M Na.sub.2 CO.sub.3 solution.

Current US Original Classification (1):
424/450

Other Reference Publication (8):
Weinstein et al.; Phase Transition Release, A New Approach to the Interaction of Proteins with Lipid Vesicles Biochim Biophys. Acta. 647:270-284 (1981).

CLAIMS:

1. A liposome containing an active agent entrapped within the liposome interior space, said liposome having a gel-phase lipid bilayer membrane comprising phospholipid which is dipalmitoylphosphatidylcholine (DPPC) and lysolipid which is monopalmitoylphosphatidylcholine (MPPC), wherein phospholipid and lysolipid are contained in the bilayer membrane in a ratio of from 99:10 to 80:20 by molar weight and wherein phospholipids are the primary lipid source for the lipid bilayer membrane and wherein lysolipid is contained in the bilayer membrane in an amount sufficient to increase the percentage of active agent released at the phase transition temperature compared to that which would occur in the absence of said lysolipid.

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